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Highlights

- Persister cells are metabolically active and have a phenotype adapted to stress protection.
- Perturbations of metabolic homeostasis might be the common, basic process leading to persister formation.
- Feedback mechanisms stabilize the cells in the persister state.
- The cellular regulation of resource allocation might be a suitable target to combat persisters.

Abstract

In recent years, our understanding about bacterial persistence has significantly advanced: we comprehend the persister phenotype better, more triggers for persistence entry have been found, and more insights in the involvement and role of toxin-antitoxin systems and other molecular mechanisms have been unravelled. In this review, we attempt to put these findings into an integrated, system-level perspective. From this point of view, persistence can be seen as a response to a strong perturbation of metabolic homeostasis, either triggered environmentally, or by means of intracellular stochasticity. Metabolic-flux-regulated resource allocation ensures stress protection, and several feedback mechanisms stabilize the cells in this protected state. We hope that this novel view can advance our understanding about persistence.

Introduction

Bacteria can survive antibiotic treatment because they either have acquired genetic resistance, allowing for growth in the presence of an antimicrobial compound, or because they express the so-called persister phenotype. Persistence is a state characterized by a transient, non-inheritable antibiotic tolerance, typically associated with dormancy and lack of antibiotic target activity. Cells in this state - persister cells - are a health threat because they can wake up and cause post-treatment relapse of infections [1]. Although persisters were discovered already in 1944 [2], most research on these cells was done in the last decade, after it was found that persisters are phenotypic variants in clonal populations [3].

Next to the persisters occurring in exponentially growing cultures at frequencies of about 10^{-6}-10^{-4} [4], several other laboratory models are currently used to study persisters: persisters formed upon starvation [5], persisters formed after overexpression of toxins [6], and persisters formed in response to a diauxic [7] or a rapid nutrient shift [8] (Fig. 1A-D). Recently, our knowledge about persistence has significantly increased, and several reviews covered this advance [9-12] and also highlighted a number of inconsistencies in the field [13].

In this current opinion review, we would like to contribute a system-level perspective on persistence, focussed on the following questions: (i) Is the persister state and the associated antibiotic tolerance achieved through active mechanisms, or is the tolerance simply based on the lack of antibiotic target activity? (ii) Is there a common mechanism, which underlies persister formation, despite the fact that persistence can be formed in so many different ways? (iii) Can the very fragmented knowledge about persisters be integrated into a system-level picture that globally explains persistence and its emergence? Drawing on the findings from the past few years, we sketch potential answers to these questions. We hope that this review and the outlined system-level perspective will stimulate the discussion and further research on bacterial persistence.

Persistence is a general stress response

Until recently, the persister phenotype was elusive. In fact, on the basis of the observation that starved cells also exhibit antibiotic tolerance [14,15], one could argue that persistence is not an actively achieved phenotype with specific features, but that the antibiotic tolerance is just conferred by the inactivity of the antibiotic targets. While experimental assessment of the persister phenotype has been difficult due to the low occurrence of persister cells in nutrient-rich cultures, early genetic screenings had identified that toxin-antitoxin systems (TAS) can increase the fraction of persisters [10,16], which provided the first hint that the persister state could indeed be an actively achieved phenotype.

System-level transcriptome studies, performed on stochastically induced persisters separated by cell sorting, or enriched by lysis of growing cells with ampicillin, revealed that the persister phenotype is characterized by a higher expression of stress response and TAS genes, as well as lower expression of flagellar genes compared to growing cells [17,18]. Further, the nutrient-shift method for generating large amounts of persisters also enabled their phenotypic characterization with other high throughput methods. Here, it was found that the persister proteome is characterized by a shift towards catabolism and a global stress response, mainly governed by the stress response regulator σ^S [19]. Moreover, metabolic and physiological analyses demonstrated that persisters take up available carbon sources and metabolize them, and grow very slowly through a respiratory metabolism [6,19,20].

Consistent with findings that dormancy is not necessary or sufficient for antibiotic tolerance [21], the persister phenotype actively mediates the enhanced tolerance of persister cells to drugs. For instance, the higher abundance of energy-dependent efflux pumps in persisters and the availability of energy in persisters [19] could enable drug efflux activity. Indeed, higher efflux activity was found to mediate the enhanced drug tolerance in persisters [22]. Thus, persistence is indeed a phenotype with a regulatory programme behind it, characterized by very slow growth, stress protection, and a

metabolism geared towards energy production, with the observed antibiotic tolerance being - at least partially - actively conveyed.

Metabolic flux limitation is at the core of persister formation

Persistence can be triggered by an array of factors. These factors include genes with pleiotropic effects such as *rpoS, relA, spoT, dksA, ssrA* and *lon*, toxin/antitoxin-system-connected genes and about 30 metabolic genes in *Escherichia coli* (cf. reviews of [12] and [11]). Further, also several environmental conditions were found to induce persistence such as internalization of bacteria by macrophages [23], sub-lethal concentrations of antibiotics and oxidative stress [24], limitation of glucose availability by addition of glucose transport inhibitor [4] and diauxic [7] or rapid nutrient shifts [8]. The fact that such a multitude of factors can induce persistence suggests that there is no single 'persister gene', but that the phenotype of persistence must rather be accomplished by system-level mechanisms.

While so far, the prevalent notion is that toxin-antitoxin systems are the key players in persister formation [9], a body of evidence suggests that a strong perturbation of metabolic homeostasis, for instance, a severe reduction in metabolic flux, might be at the core of persister formation. First, as mentioned above, various metabolic genes have been shown to affect persister frequency [11]. Second, in different persistence models, it was found that a decrease in metabolic flux (sometimes inferred from a reduction in growth rate) correlated with an increased persister frequency. For instance, stochastic persister formation was found to increase with increasing concentrations of glucose transport inhibitor, which caused a reduction in growth rate [4]. Further, the frequency of nutrient shift-induced persisters was found to be dependent on metabolic flux, both before and after the nutrient shift [8,19]. Moreover, it was shown that in *S. aureus* persistence is caused by a decrease in ATP concentration [25], a manifestation of a perturbed metabolic homeostasis. Finally, it was shown that imbalances in the metabolic network other than metabolic flux limitation can cause persister formation: following the finding that the fastest-growing sub-population of cells in an

isogenic bacterial population was enriched in persister cells [21], another study computationally predicted and then experimentally verified that growth arrest and persistence can be caused by imbalances in the metabolic network, other than those initiated by metabolic flux limitations [26]. According to this study, if some metabolic reactions occur at rates that are too fast for optimality, metabolites could accumulate, and toxicity of these metabolites can cause growth arrest, mediating persistence.

All strong perturbations of metabolism, whether coming from environmental perturbations, or from intracellular stochastic variation in enzyme levels [27], which finally cause the metabolic flux to collapse, could theoretically lead to a vicious cycle ultimately leading to a state of non-growth: A reduction in metabolic flux inherently reduces the rate protein synthesis. If a reduction of metabolic flux would be so strong that the resulting protein synthesis rate would be lower than the inherent protein degradation rate, then this could result in a vicious cycle (Fig. 2A), where metabolic homeostasis, i.e. normal metabolic flux, could not be restored anymore, because proteins would degrade faster than they are synthesized. Indeed, limiting protein synthesis has been shown to induce persistence [28].

The end point of such theoretical vicious cycle would be a state of non-growth. Such a state – which notably would not (!) be the persister state - could have existed in very primitive organisms that had not yet evolved the stress response. This state of non-growth (without stress response) and the normal growth state could be considered two different attractors on a phenotypic landscape (Fig. 2B). Upon a weak perturbation of metabolic homeostasis, mechanisms, such as the transcriptional circuits cAMP-CRP and FBP-Cra in *E. coli*, might kick in and restore the normal growth state. Upon too strong perturbations, the vicious cycle, however, might pull the cell to the non-growth attractor. Metabolic flux could be the critical variable in this decision-making process: Not only the entry into the vicious cycle might be metabolic-flux dependent, but also the mechanisms to restore the normal growth state were found to be partly regulated by metabolic flux [8,29,30].

If a cell would get into a situation of such a vicious cycle, where proteins degrade faster than they are synthesized, then there would be no more way to restore the metabolic homeostasis. These cells, while transiently tolerant to antibiotics due to lack of antibiotic target activity, would be devoid of energy and resources, and essentially be doomed to die, unless very favourable environmental changes would occur soon enough. To avoid such a situation, we argue that cells have evolved another attractor, the safe persister state (Fig. 2C). Here, the still available (but limited) external or internal resources after a perturbation of metabolic homeostasis are used to ensure the reaching of the safe and protected state of persistence, for instance by using the limited resources for the expression of stress response mechanisms. These mechanisms pull the cell away from the non-growth attractor, thus avoiding death.

Flux-dependent resource allocation ensures safe shut-down

We argue that in order to avoid being pulled into the vicious cycle and essentially into death, cells, with sensing increasingly worse metabolic operation, redirect increasingly more protein expression resources into stress protection, such that they can reach the safe persister state with high probability, should conditions get even worse. In fact, recent *E. coli* proteome studies showed that cellular resource allocation is indeed done in a flux- or growth rate-dependent manner [31,32]. Analyses of the acquired proteome data with genome-scale models of metabolism and macromolecular expression (ME models) showed that *E. coli* already under normal growth conditions preventatively invests significant resources for stress protection [33,34].

Which mechanism could be responsible for allocating resources to the stress proteome in a growth-rate or flux-dependent manner? Here, the alarmone ppGpp (guanosine tetraphosphate), triggering transcriptional programmes such as the stringent response and the σ^S (RpoS) stress response [35], might be responsible for the allocation of resources into the stress proteome. In fact, it was found that the above mentioned allocation of protein expression resources into stress protection is mainly regulated by the general stress response sigma factor σ^S [33]. Also, it was shown in *E. coli* that

investment of resources in stress response depends on ppGpp concentration [36]. Further, while ppGpp concentration increases in response to amino-acid starvation [37,38], it was also found to correlate inversely with the growth rate [39], implying that its concentration is flux-dependent. Also, in persisters, where metabolic flux drops dramatically, the intracellular ppGpp concentration was shown to be increased: In stochastically-formed persister cells, an increased ppGpp concentration was inferred from fluorescent reporters under the control of promoters that were previously found to be either repressed or induced after an increase in ppGpp concentration [4]. Moreover, in persisters generated through rapid nutrient shifts intracellular ppGpp levels were directly quantified and found to be elevated, compared to cells growing on glucose [19]. Thus, even though the regulation mechanisms responsible for ppGpp concentrations remain elusive (Gaca et al., 2015), ppGpp could be responsible for allocating resources towards stress protection in a gradual, flux-dependent manner.

If resources are allocated for stress protection, this means that at the same time less of these resources are available for restoring the normal growth state. Therefore, a higher allocation of resources into stress protection must increase the likelihood for cells to fall towards the persistence attractor, rather than the growth attractor. Indeed, it was shown that overexpression of *rpoS* – a downstream target of ppGpp [40] – significantly increases the fraction of cells entering persistence upon nutrient-shifts, with the fraction correlating with the *rpoS* induction level [19]. Moreover, strains not producing ppGpp, putatively responsible for the stress resource allocation, were found to have decreased number of persisters, yet, they were still observed [5,41]. It remains to be tested, whether these ppGpp negative cells have the specific features of the persister phenotype, or whether such cells, because of the lack of stress protection, would rather resemble the cells of a non-growth state (Fig. 2C).

We suggest that flux-dependent mechanisms increase the intracellular ppGpp concentration in response to metabolic flux limitation, which subsequently triggers the RpoS-mediated general stress

response. This response ensures that the resources are allocated such that the safe persister state can be reached, if necessary.

Additional mechanisms enhance and stabilize persistence

The resource allocation ensures that the cells reach the safe state of persistence after a perturbation of metabolic homeostasis, instead of entering the vicious cycle, the state of non-growth, which could eventually result in cell death. On top of these resource allocation mechanisms, there are additional mechanisms that stabilize the cells in the persister state. For instance, ppGpp inhibits growth via various mechanisms, for instance, by affecting ribosome assembly [42], by inhibiting transcription initiation through direct binding to the RNA polymerase [43], or through induction of TAS [4,44-46]. Many TAS rely on a mixed-feedback motif, combining transcriptional regulation and protein-protein interactions, and thus are inherently capable of causing the growth - persistence bistability [47]. TA activity can also be influenced without direct regulation, via growth-rate related effects on protein synthesis dynamics. A general growth-rate dependent mechanisms of activation of a bistable toxin-antitoxin systems has been suggested [48]. Another study has shown that coupling of free toxin concentration to the growth rate (and the dilution rate, but not protein synthesis dynamics) can mediate HipAB toxin-antitoxin system bistability, causing persistence [46].

However, toxins inhibit mechanisms that are crucial for growth, but they are not directly responsible for resource allocation programming. If so, what is their role in bacterial persistence? In our opinion, TAS could be directly involved in the process of making a decision after a perturbation of metabolic homeostasis: should the cell enter persistence, or try to restore the growth state? In theory, by inhibiting basic cellular processes, such as transcription, translation or replication, leading to growth arrest, these systems would in turn reduce metabolic flux (by decreasing the demand for biomass building blocks), which would cause ppGpp accumulation and ultimately also trigger persistence. Recent findings indeed point towards this direction: it was found that the toxin HipA causes the accumulation of uncharged $tRNA^{Glu}$ through inhibition of the GltX (a glutamate-tRNA ligase), which

198 triggers the RelA-dependent ppGpp accumulation [45,49]. The trigger for HipA action is thought to
199 be amino acid starvation, which leads to ppGpp accumulation [49]. Because HipA activity is both
200 caused by and causes ppGpp accumulation, it creates a positive feedback. Moreover, it has been
201 shown that TAS indeed modulate ppGpp levels under amino acid starvation through regulation of
202 global mRNA levels, which in turn regulate the RelA mediated ppGpp synthesis [50]. Simply put, the
203 effects that TAS elicit in the cell are the same as the triggers that made these TAS active in the first
204 place, establishing a positive feedback.

205 Although ectopic overexpression of TAS can increase the number of persisters [17,51-53], we think
206 that without ectopic induction, the ability of various TAS to be induced at different ppGpp threshold
207 concentrations, or in response to other cues specific to various environmental perturbations, would
208 only serve the purpose of fine-tuning the decision-making process of entering persistence.

209 An alternative, or an additional function of TAS could be to stabilize the persister state once it is
210 established, i.e. after the flux-dependent decision has already been made. In this scenario, TAS
211 would prevent cells from waking up, rather than directly cause persistence. Indeed, while TAS
212 deletion has been shown to influence the fraction of persisters in growing populations [4,44,54], the
213 number of persisters does not change greatly compared to the total number of cells in the culture.
214 Also, TAS were shown to be of minor importance for the formation of nutrient-shift induced
215 persisters [19] and *hipA* overexpression led to prolonged wake-up times of persister cells [55].
216 Furthermore, a computational study, investigating the dynamics of phenotype switching, found that
217 in exponentially growing cultures, in which persisters are stochastically formed, the fraction of
218 persisters is not influenced significantly by modulating the wake-up rate [56]. Together, these
219 findings would support the notion that TAS are rather important to prevent the exit from
220 persistence. However, it remains to be ultimately proven that TAS are in place to primarily stabilize
221 the persister state and to enhance the growth-inhibiting feedback, initiated by a strong perturbation
222 of metabolic homeostasis, rather than to trigger persistence.

Persistence as a robust biological system

Taken together, we propose that the states of persistence or growth can be viewed as two attractors in a phenotypic landscape with 'metabolic flux' as one dimension and 'activity of growth inhibiting mechanisms' as second dimension, and a watershed separating the two attractors (Fig. 3A-C). Once a metabolic perturbation occurs, the multiple layers of regulation decide between a trade-off: growth restoration at the risk of death, or stress protection and persistence at the cost of no growth. In constant environmental conditions, stochastic induction of growth-inhibiting mechanisms or a stochastically low expression of a flux-controlling enzyme could force a cell to enter the persister state (Fig. 3A). Upon increasing the activity of growth-inhibiting and stress protection mechanisms (for example, after *rpoS* overexpression), cells would transit into persistence already at higher metabolic flux (Fig. 3B). Consistently, increased RpoS levels [19] and TAS overexpression [17,51-53] increases the number of persisters. On the other hand, a strong decrease in metabolic flux would mediate persistence with only subsequent induction of the stress response (Fig. 3C), which is consistent, for instance, with the finding that sudden nutrient shifts inducing persistence.

While persistence can be induced through perturbing the cells along either of the two axes (Fig. 3A-C), these two variables are also inherently tethered and a change in one parameter will inevitably influence the other: On one hand, growth inhibition (for instance, through TAS action) reduces the metabolic flux by decreasing the demand for biomass building blocks. On the other hand, flux limitation can cause increased ppGpp concentrations, which in turn induce growth inhibiting mechanisms [35], or the TAS activation mediated directly through growth-rate-decrease related effects [46,48]. Thus, the two axes in Figs. 3A-C are not truly independent.

The system that mediates persistence resembles the generic robust system as outlined by Kitano [57]: The state of persistence is a phenotype achieved and maintained via complex and nested regulation mechanisms. Various environmental perturbations can trigger persistence. The metabolic flux at the core of persistence is connected to the various inputs through mechanisms that react to

metabolic perturbations and cause, for instance, the flux-dependent ppGpp accumulation. The particular mechanisms mediating the persister phenotype are connected to the metabolic core through ppGpp-dependent resource allocation regulator (stringent response, RpoS), through ppGpp mediated inhibition of basic cellular processes (TAS), or through growth-rate-dependent effects that are not ppGpp-dependent (Fig. 4). The redundancy found in the system that mediates persistence would cause a high robustness of this system towards genetic modifications, which manifests itself in the inability to eradicate persisters with simple genetic approaches. On the other hand, the characteristic topology of the regulation having metabolic flux at its core would imply a high fragility of persister cells towards metabolic perturbations, which target the central node of the system. This fragility would manifest itself in the ease of generating persister cells by limiting metabolic flux, as well as the ease of waking them up by restoring the metabolic flux.

How to target persistence?

Following the logic of the outlined systems perspective on persisters, combatting persisters could be achieved in two ways. One possibility would be to induce persister exit, which can be accomplished by restoration of metabolic flux [19,58]. Another strategy would be to manipulate the cellular resource allocation, i.e. preventing the cellular stress response, which was shown to pull cells into the persistence state. Being less stress protected would likely not only compromise the persisters' long-term survival, but it would also increase the probability that persisters would try to restore their normal phenotype (i.e. moving towards the growth attractor). In case a cell manages to restore the normal growth phenotype, it would again be susceptible to antibiotics. In case, such restoration attempt fails, this would likely result in cell death. Indeed, it was shown that bacteria lacking the stress regulator *rpoS* form less persisters [5,19] and that lack of ppGpp greatly diminished the formation of both ampicillin and ofloxacin persisters [41]. Thus, targeting the ppGpp-mediated stress response also seems to be a potential strategy for combatting persisters.

Conclusions

The current and most prevalent view on persisters is centred on toxin-antitoxin systems [9]. Despite their important role in persistence, in this review, we aimed to highlight the role of metabolism in persister cell formation and to provide a broader view on persistence. We hope that through this perspective we can further advance our understanding of bacterial persistence. In fact, we feel that our view, and the expressed importance of metabolism, provides answers to the often observed sensitivity of persisters to laboratory growth conditions and for the inconsistencies between results obtained by different research groups, as highlighted in the recent reviews [9,10,13].

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Figures

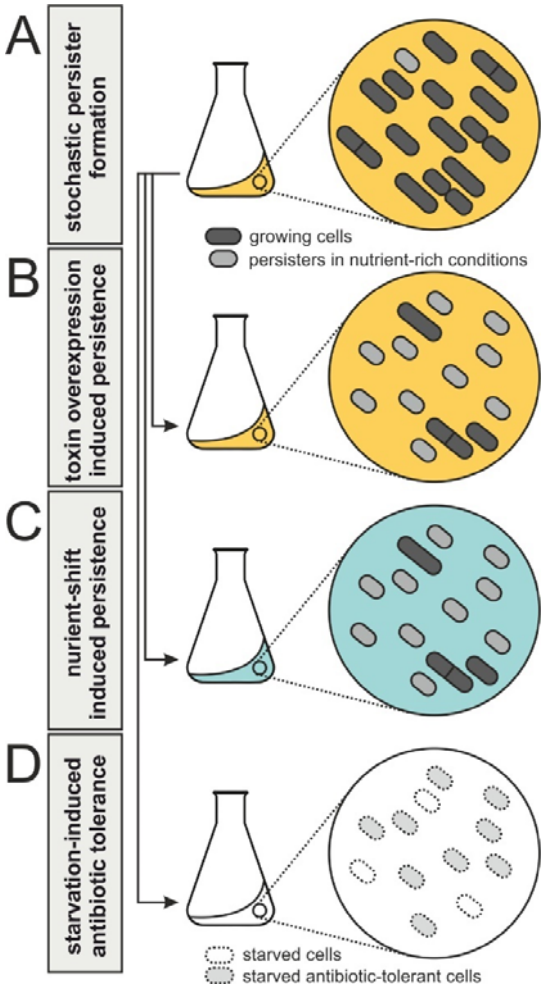
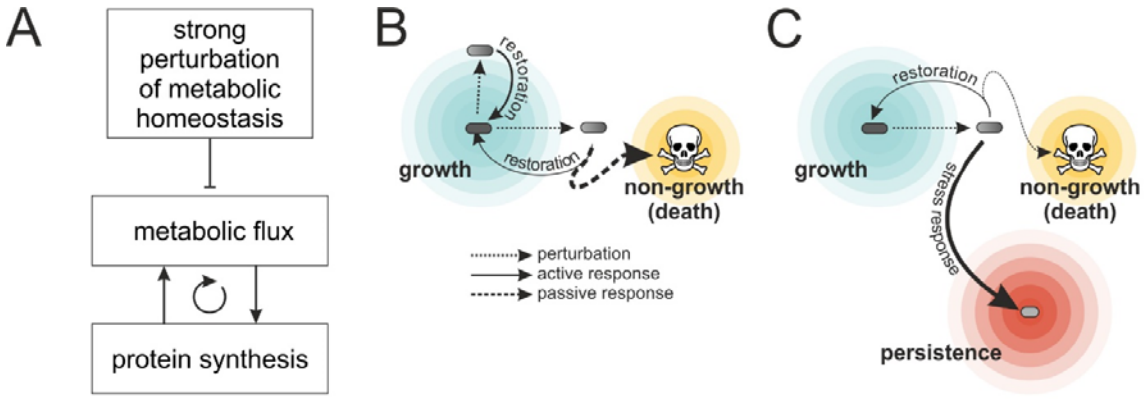


Figure 1 - Various laboratory models used for generation of persisters and antibiotic-tolerant cells. **A -** In exponentially growing cultures, persisters can be formed stochastically at low frequencies. **B** - Induction of toxins can also increase the number of persisters. **C** - Certain gradual and sudden nutrient shifts of exponentially growing cultures can generate populations with significant fractions of persister cells, present in nutrient-rich conditions. **D** - Upon starvation, large amounts of antibiotic-tolerant cells are generated. However, these cells reside in nutrient-deprived conditions and might not be able to fully express the persister phenotype.



296

297 **Figure 2 - Growth, non-growth and persistence can be considered as attractors on a phenotypic**
298 **landscape. A -** A limitation in metabolic flux causes slowing down or inhibition protein synthesis,
299 which in extreme cases can prevent metabolic adaptation and restoration of the metabolic flux,
300 which got perturbed. This creates feedback, and a vicious cycle causing a stable state of non-growth.
301 **B -** The primitive vicious cycle creates two basic attractors - growth and non-growth (death). A weak
302 externally induced or stochastically occurring metabolic perturbation could be restored and the cell
303 returns to the growth state. However, upon strong perturbations, the cell might enter the vicious
304 cycle and die. **C** – Next to growth, persistence is an additional attractor that evolved next to the non-
305 growth state (death). Through activation of stress response mechanisms, upon strong perturbations,
306 the limited resources are directed into stress protection rather than risky restoration of growth that
307 could result in death.

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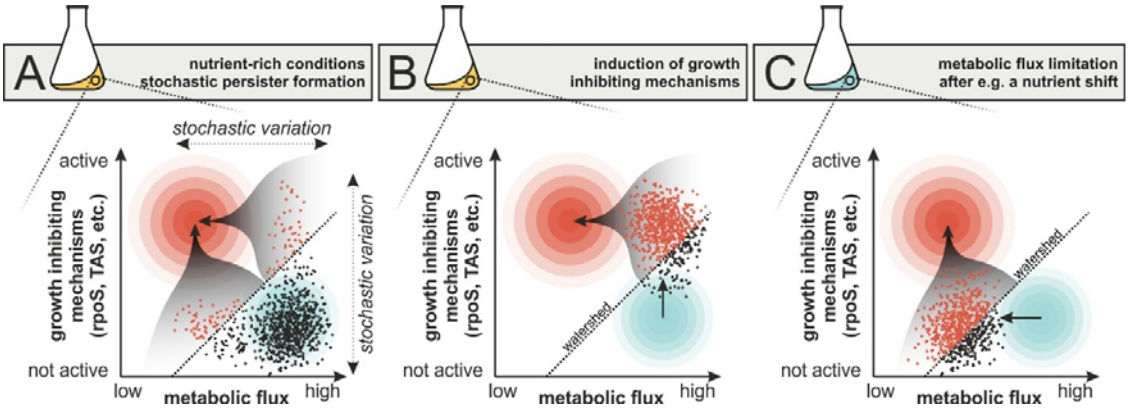

Figure 3 - The position of a cell on the phenotypic landscape determines its fate. The landscape is defined by the magnitude of metabolic flux and activity of growth-inhibiting mechanisms. The states of persistence and normal growth are separated by a watershed. **A -** During normal, nutrient-unlimited growth some cells, for stochastic reasons, might experience high activity of growth inhibiting mechanisms, forcing them into the persister state. Alternatively, a stochastically induced flux limitation, can also pull cells into persistence under normal growth conditions. **B -** Upon induction of growth inhibiting mechanisms (such as TAS), cells with higher activity of such mechanisms are pushed beyond the watershed and enter persistence, even at high metabolic fluxes. **C –** Strong metabolic perturbations leading to a drop in metabolic fluxes can pulls cells into the persister state.

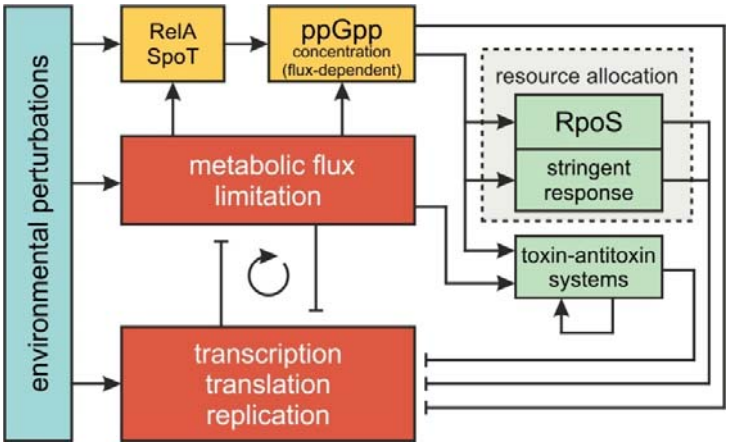

Figure 4 - The system-level view on persistence, its complexity and topology. Multiple inputs can trigger persistence, and multiple mechanisms are responsible for enhancing the feedback in the primitive vicious cycle (cf. Fig. 2A). At the core of the system governing persistence is metabolic flux. A limitation in metabolic flux, caused by an environmental perturbation, either directly via growth rate or through the increased, flux-dependent ppGpp concentration, with the help of resource allocation, inhibits the basic cellular processes: transcription, translation and replication. The growth inhibition caused by these effectors further limits the metabolic flux by reducing the demand for building blocks, enhancing the feedback established by the primitive vicious cycle.

References

[1] Cohen N, Lobritz M, Collins J: **Microbial Persistence and the Road to Drug Resistance.** *Cell Host & Microbe* 2013, **13**: 632-642.

[2] Bigger J: **Treatment of Staphylococcal Infections with Penicillin by Intermittent Sterilisation.** *The Lancet* 1944, **244**: 497-500.

[3] Balaban N, Merrin J, Chait R, Kowalik L, Leibler S: **Bacterial persistence as a phenotypic switch.** *Science* 2004, **305**: 1622-1625.

[4*] Maisonneuve E, Castro-Camargo M, Gerdes K: **(p)ppGpp Controls Bacterial Persistence by Stochastic Induction of Toxin-Antitoxin Activity.** *Cell* 2013, **154**: 1140-1150.

This paper shows the mechanisms through which ppGpp activates TAS, triggering persistence.

[5] Dao Nguyen, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, McKay G, Siehnel R, Schafhauser J, Wang Y*, et al.*: **Active Starvation Responses Mediate Antibiotic Tolerance in Biofilms and Nutrient-Limited Bacteria.** *Science* 2011, **334**: 982-986.

[6] Mok WWK, Park JO, Rabinowitz JD, Brynildsen MP: **RNA Futile Cycling in Model Persisters Derived from MazF Accumulation.** *Mbio* 2015, **6**: e01588-15.

[7**] Amato SM, Orman MA, Brynildsen MP: **Metabolic Control of Persister Formation in Escherichia coli.** *Mol.Cell* 2013, **50**: 475-487.

This paper shows involvement of metabolism in persister formation and shows that diauxic shifts are a source of persisters.

[8**] Kotte O, Volkmer B, Radzikowski JL, Heinemann M: **Phenotypic bistability in Escherichia coli's central carbon metabolism.** *Molecular systems biology* 2014, **10**: 736.

This paper introduces the sudden nutrient-shifts as a mean to generate persisters and shows that metabolic flux is key to persister formation.

[9] Harms A, Maisonneuve E, Gerdes K: **Mechanisms of bacterial persistence during stress and antibiotic exposure.** *Science* 2016, **354**.

[10] Brauner A, Fridman O, Gefen O, Balaban NQ: **Distinguishing between resistance, tolerance and persistence to antibiotic treatment.** *Nature reviews.Microbiology* 2016, **14**: 320-30.

[11] Amato SM, Fazen CH, Henry TC, Mok WWK, Orman MA, Sandvik EL, Volzing KG, Brynildsen MP: **The role of metabolism in bacterial persistence.** *Front.Microbiol.* 2014, **5**: 70.

[12] Kaldalu N, Hauryliuk V, Tenson T: **Persisters - as elusive as ever.** *Appl.Microbiol.Biotechnol.* 2016, **100**: 6545-6553.

[13] Kim J, Wood TK: **Persistent Persister Misperceptions.** *Frontiers in Microbiology* 2016, **7**: 2134.

[14] Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, McKay G, Siehnel R, Schafhauser J, Wang Y*, et al.*: **Active Starvation Responses Mediate Antibiotic Tolerance in Biofilms and Nutrient-Limited Bacteria.** *Science* 2011, **334**: 982-986.

[15] Fung DKC, Chan EWC, Chin ML, Chan RCY: **Delineation of a Bacterial Starvation Stress Response Network Which Can Mediate Antibiotic Tolerance Development.** *Antimicrob.Agents Chemother.* 2010, **54**: 1082-1093.

[16] Page R, Peti W: **Toxin-antitoxin systems in bacterial growth arrest and persistence.** *Nature Chemical Biology* 2016, **12**: 208-214.

[17] Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, Lewis K: **Persisters: a distinct physiological state of E-coli.** *Bmc Microbiology* 2006, **6**: 53.

[18] Keren I, Shah D, Spoering A, Kaldalu N, Lewis K: **Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli.** *J.Bacteriol.* 2004, **186**: 8172-8180.

[19**] Radzikowski JL, Vedelaar S, Siegel D, Ortega ÁD, Schmidt A, Heinemann M: **Bacterial persistence is an active σ^S stress response to metabolic flux limitation.** *Mol Syst Biol* 2016, **12**.

This paper shows that persisters are metabolically active and provides the first dynamic and global characterization of the persister phenotype.

[20*] Orman MA, Brynildsen MP: **Inhibition of stationary phase respiration impairs persister formation in E-coli.** *Nature Communications* 2015, **6**: 7983.

This paper shows that energy availability mediates enhanced persistence, supporting the notion that persistence is an active state.

[21] Orman MA, Brynildsen MP: **Dormancy Is Not Necessary or Sufficient for Bacterial Persistence.** *Antimicrob.Agents Chemother.* 2013, **57**: 3230-3239.

[22] Pu Y, Zhao Z, Li Y, Zou J, Ma Q, Zhao Y, Ke Y, Zhu Y, Chen H, Baker MAB*, et al.*: **Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells.** *Mol.Cell* 2016, **62**: 284-294.

[23] Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW: **Internalization of Salmonella by Macrophages Induces Formation of Nonreplicating Persisters.** *Science* 2014, **343**: 204-208.

[24] Wu Y, Vulic M, Keren I, Lewis K: **Role of Oxidative Stress in Persister Tolerance.** *Antimicrob.Agents Chemother.* 2012, **56**: 4922-4926.

[25*] Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, Clair G, Adkins JN, Cheung AL, Lewis K: **Persister formation in Staphylococcus aureus is associated with ATP depletion.** *Nature Microbiology* 2016, **1**: 16051.

This paper extends the notion that metabolism is important for persister formation to other species.

[26] Ray JCJ, Wickersheim ML, Jalihal AP, Adeshina YO, Cooper TF, Balazsi G: **Cellular Growth Arrest and Persistence from Enzyme Saturation.** *Plos One* 2016, **11**: e1004825.

[27] Kiviet DJ, Nghe P, Walker N, Boulineau S, Sunderlikova V, Tans SJ: **Stochasticity of metabolism and growth at the single-cell level.** *Nature* 2014, **514**: 376-+.

[28] Kwan BW, Valenta JA, Benedik MJ, Wood TK: **Arrested Protein Synthesis Increases Persister-Like Cell Formation.** *Antimicrob.Agents Chemother.* 2013, **57**: 1468-1473.

[29] Kotte O, Zaugg JB, Heinemann M: **Bacterial adaptation through distributed sensing of metabolic fluxes.** *Molecular Systems Biology* 2010, **6**: 355.

[30] Hermsen R, Okano H, You C, Werner N, Hwa T: **A growth-rate composition formula for the growth of E. coli on co-utilized carbon substrates.** *Mol.Syst.Biol.* 2015, **11**.

[31] Schmidt A, Kochanowski K, Vedelaar S, Ahrne E, Volkmer B, Callipo L, Knoops K, Bauer M, Aebersold R, Heinemann M: **The quantitative and condition-dependent Escherichia coli proteome.** *Nat.Biotechnol.* 2016, **34**: 104-110.

[32] Hui S, Silverman JM, Chen SS, Erickson DW, Basan M, Wang J, Hwa T, Williamson JR: **Quantitative proteomic analysis reveals a simple strategy of global resource allocation in bacteria.** *Molecular Systems Biology* 2015, **11**.

[33*] Yang L, Yurkovich JT, Lloyd CJ, Ebrahim A, Saunders MA, Palsson BO: **Principles of proteome allocation are revealed using proteomic data and genome-scale models.** *Scientific Reports* 2016, **6**: 36734.

This paper shows that cells already at normal growth conditions invest significant protein resources into stress response.

[34] O'Brien EJ, Utrilla J, Palsson BO: **Quantification and Classification of E-coli Proteome Utilization and Unused Protein Costs across Environments.** *Plos Computational Biology* 2016, **12**: e1004998.

[35] Gaca AO, Colomer-Winter C, Lemos JA: **Many Means to a Common End: the Intricacies of (p)ppGpp Metabolism and Its Control of Bacterial Homeostasis.** *J.Bacteriol.* 2015, **197**: 1146-1156.

[36**] Traxler MF, Zacharia VM, Marquardt S, Summers SM, Nguyen H, Stark SE, Conway T: **Discretely calibrated regulatory loops controlled by ppGpp partition gene induction across the "feast to famine" gradient in *Escherichia coli*.** *Mol.Microbiol.* 2011, **79**: 830-845.

This paper show that ppGpp drives resource allocation in a concentration-dependent manner.

[37] Haseltine W, Block R: **Synthesis of Guanosine Tetraphosphate and Pentaphosphate Requires Presence of a Codon-Specific, Uncharged Transfer Ribonucleic-Acid in Acceptor Site of Ribosomes - (Stringent Control Ppgpp (Msi) and Pppgpp (Msii) Protein Synthesis Escherichia-Coli).** *Proc.Natl.Acad.Sci.U.S.A.* 1973, **70**: 1564-1568.

[38] Payoe R, Fahlman RP: **Dependence of RelA-Mediated (p)ppGpp Formation on tRNA Identity.** *Biochemistry (N.Y.)* 2011, **50**: 3075-3083.

[39] Lazzarini R, Cashel M, Gallant J: **Regulation of Guanosine Tetraphosphate Levels in Stringent and Relaxed Strains of Escherichia-Coli.** *J.Biol.Chem.* 1971, **246**: 4381-+.

[40] Durfee T, Hansen A, Zhi H, Blattner FR, Jin DJ: **Transcription Profiling of the Stringent Response in Escherichia coli.** *Journal of Bacteriology* 2008, **190**: 1084-1096.

[41*] Amato SM, Brynildsen MP: **Persister Heterogeneity Arising from a Single Metabolic Stress.** *Current Biology* 2015, **25**: 2090-2098.

This paper showed that ppGpp modulates the formation of both ampicillin and ofloxacin persisters, and focusses on persister heterogeneity as an aspect that is not covered in this review.

[42] Corrigan RM, Bellows LE, Wood A, Gruendling A: **ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria.** *Proc.Natl.Acad.Sci.U.S.A.* 2016, **113**: E1710-E1719.

[43] Ross W, Vrentas CE, Sanchez-Vazquez P, Gaal T, Gourse RL: **The Magic Spot: A ppGpp Binding Site on E. coli RNA Polymerase Responsible for Regulation of Transcription Initiation.** *Mol.Cell* 2013, **50**: 420-429.

[44] Maisonneuve E, Shakespeare LJ, Jorgensen MG, Gerdes K: **Bacterial persistence by RNA endonucleases.** *Proc.Natl.Acad.Sci.U.S.A.* 2011, **108**.

[45] Germain E, Castro-Roa D, Zenkin N, Gerdes K: **Molecular Mechanism of Bacterial Persistence by HipA.** *Mol.Cell* 2013, **52**: 248-254.

[46] Feng J, Kessler DA, Ben-Jacob E, Levine H: **Growth feedback as a basis for persister bistability.** *Proc.Natl.Acad.Sci.U.S.A.* 2014, **111**: 544-549.

[47] Cataudella I, Sneppen K, Gerdes K, Mitarai N: **Conditional Cooperativity of Toxin - Antitoxin Regulation Can Mediate Bistability between Growth and Dormancy.** *Plos Computational Biology* 2013, **9**: e1003174.

[48] Klumpp S, Zhang Z, Hwa T: **Growth Rate-Dependent Global Effects on Gene Expression in Bacteria.** *Cell* 2009, **139**: 1366-1375.

[49**] Kaspy I, Rotem E, Weiss N, Ronin I, Balaban NQ, Glaser G: **HipA-mediated antibiotic persistence via phosphorylation of the glutamyl-tRNA-synthetase.** *Nature Communications* 2013, **4**: 3001.

This paper showed that HipAB TAS is triggered by nutrient limitation via ppGpp, and emphasises the role of TAS for stabilizing the cells in the persister state.

[50] Tian C, Roghanian M, Jorgensen MG, Sneppen K, Sorensen MA, Gerdes K, Mitarai N: **Rapid Curtailing of the Stringent Response by Toxin-Antitoxin Module-Encoded mRNases.** *J.Bacteriol.* 2016, **198**: 1918-1926.

[51] Vazquez-Laslop N, Lee H, Neyfakh A: **Increased persistence in Escherichia coli caused by controlled expression of toxins or other unrelated proteins.** *J.Bacteriol.* 2006, **188**: 3494-3497.

[52] Korch SB, Hill TM: **Ectopic overexpression of wild-type and mutant hipA genes in Escherichia coli: Effects on macromolecular synthesis and persister formation.** *J.Bacteriol.* 2006, **188**: 3826-3836.

[53] Tashiro Y, Kawata K, Taniuchi A, Kakinuma K, May T, Okabe S: **RelE-Mediated Dormancy Is Enhanced at High Cell Density in Escherichia coli.** *J.Bacteriol.* 2012, **194**.

[54] Germain E, Roghanian M, Gerdes K, Maisonneuve E: **Stochastic induction of persister cells by HipA through (p)ppGpp-mediated activation of mRNA endonucleases.** *Proc.Natl.Acad.Sci.U.S.A.* 2015, **112**: 5171-5176.

[55] Rotem E, Loinger A, Ronin I, Levin-Reisman I, Gabay C, Shoresh N, Biham O, Balaban NQ: **Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence.** *Proc.Natl.Acad.Sci.U.S.A.* 2010, **107**: 12541-12546.

[56] Patra P, Klumpp S: **Population Dynamics of Bacterial Persistence.** *Plos One* 2013, **8**: e62814.

[57] Kitano H: **Biological robustness.** *Nat.Rev.Genet.* 2004, **5**: 826-837.

[58] Orman MA, Brynildsen MP: **Establishment of a method to rapidly assay bacterial persister metabolism.** *Antimicrob.Agents Chemother.* 2013, **57**: 4398-409.